

Supplementary Information

S1 Materials and Methods

S1.1 Animals

All animals were handled according to the Guidelines for the Care and Use of Laboratory Animals established by the Beijing Association for Laboratory Animal Science. Animal experiments were approved by the Animal Ethics Committee of College of Biological Sciences, China Agricultural University.

S1.2 Cas9 and sgRNA Plasmid Construction

The Cas9-coding sequence, derived from *Streptococcus pyogenes* strain M1 GAS (NC_002737.1), was codon-optimized based on the general codon usage in sheep and then synthesized (Genewiz). The synthesized sequence contained the Kozak sequence, followed by a 3×Flag tag and Cas9. In addition, two nuclear localization signal (NLS) sequences were added flanking the Cas9 coding region. The fused sequences (Kozak-Flag-NLS-Cas9-NLS) were cloned into the pcDNA3.1(+) vector through Hind III restriction site.

Two sgRNA scaffold sequences, T7-sgRNA and U6-sgRNA, were synthesized (Genewiz) and cloned into pMD-19T vector (Takara), respectively. The first was used for *in vitro* transcription and the second for cell transfection. To make these sgRNA constructs bearing customized targeting sequence, the vectors were digested with *Bbs* I restriction enzyme, and ligated with the double-strand oligonucleotides, which were annealed from a pair of synthesized oligonucleotides based on the targeting sites of *MSTN* gene. Two oligos were annealed in a thermocycler using the following parameters: 95°C for 2 min; 95°C for 1 min; ramp down to 25°C at 1°C·min⁻¹. The annealed oligonucleotides had two different overhangs that were complementary to the corresponding sticky ends of *Bbs* I-digested pMD-19T-T7/U6-sgRNA vectors to ensure directional cloning.

S1.3 Cell Culture

Sheep fetal fibroblast (SFB) cell line was cultured in Dulbecco's modified Eagle's medium (Neuronbc) containing 10% fetal bovine serum under 5% CO₂ at 37°C. The cells were seeded into 6-well plates (Thermo). After 24 h, cells were co-transfected with the mixture of two plasmids, pcDNA3.1(+)-Cas9 and pMD-19T-U6-sgRNA, with the mass ratio of 1:1 (2.5 µg per well), following the instruction of Lipofectamine 2000 (Invitrogen). Cells were harvested at 48 h post transfection.

S1.4 SURVEYOR Nuclease Assay for Genome Modification

Genomic DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen) following the manufacture's protocol. The genomic regions surrounding the CRISPR target site was PCR amplified using Q5 High-Fidelity DNA Polymerase (New England Biolabs). The PCR products were purified using E.Z.N.A Gel Extraction Kit (Omega Bio-Tek) following the manufacture's instruction. A total of 400 ng of the purified PCR products were mixed with 1 μL 10 \times LA Taq Buffer II (Mg^{2+} free) (Takara) and ultrapure water to a final volume of 10 μL and subjected to a re-annealing and enzyme digestion procedure following the procedure of Surveyor Mutation Detection Kit (Transgenomics). The heteroduplex formation re-annealing process is: 95°C for 10min, 95 to 85°C ramping at $-2^\circ\text{C}\cdot\text{s}^{-1}$, 85°C for 1min, 85 to 75°C ramping at $-0.3^\circ\text{C}\cdot\text{s}^{-1}$, 75°C for 1min, 75 to 65°C ramping at $-0.3^\circ\text{C}\cdot\text{s}^{-1}$, 65°C for 1min, 65 to 55°C ramping at $-0.3^\circ\text{C}\cdot\text{s}^{-1}$, 55°C for 1min, 55 to 45°C ramping at $-0.3^\circ\text{C}\cdot\text{s}^{-1}$, 45°C for 1min, 45 to 35°C ramping at $-0.3^\circ\text{C}\cdot\text{s}^{-1}$, 35°C for 1min, 35 to 25°C ramping at $-0.3^\circ\text{C}\cdot\text{s}^{-1}$, 25°C for 1min, hold at 4°C. After annealing, 10 μL products were treated with 2 μL MgCl_2 , 1 μL Nuclease S, 1 μL Enhancer S and 6 μL ultrapure water to a final volume of 20 μL . After incubation in a thermocycler at 42°C for 1h, products were added with 2 μL Stop Solution and analyzed on 8% TBE poly-acrylamide gels. Gels were stained with $0.5 \mu\text{g}\cdot\text{mL}^{-1}$ ethidium bromide for 30 min and imaged with Gel Logic 212 Imaging System (Kodak). Quantification was based on relative band intensities. Indel percentage was determined by the formula $100 \times (1 - (1 - (b + c)/(a + b + c))^{1/2})$, wherein a is the integrated intensity of the undigested PCR product and b and c are the integrated intensities of each cleavage product.

S1.5 Preparation of Cas9 mRNA and sgRNA

Cas9 and sgRNA coding regions containing T7 promoter were PCR amplified by TransStart FastPfu DNA Polymerase (TransGen Biotech) from each plasmid constructed above. The T7-Cas9 PCR products were gel purified and used as the template for *in vitro* transcription (IVT) using mMACHINE T7 ULTRA Transcription Kit (Life Technologies). The poly (A) tailing reaction was performed after the completion of capping using Poly (A) Tailing Kit (Life Technologies) according to the manufacturer's instruction. The T7-sgRNA PCR product was gel purified and used as the template for IVT using MEGAscript T7 Kit (Life Technologies). The sgRNA was purified by ethanol precipitation and the Cas9-encoding mRNA was by lithium chloride (LiCl) precipitation. All the products were re-dissolved in RNase-free water and stored at -80°C .

S1.6 Microinjection of Zygotes and Embryo Transfer

The sheep zygotes were obtained by super-ovulation of females and artificial insemination. The zygotes were flushed using sterile filtered embryo flushing solution (Emcare) from the oviduct of the sheep. After that, 2–5 pL TE solution containing $50 \text{ ng}\cdot\mu\text{L}^{-1}$ of sgRNA and $100 \text{ ng}\cdot\mu\text{L}^{-1}$ of Cas9 mRNA were injected into the cytoplasm of pronucleus embryos using injection needles. Injections were performed by an Eppendorf transferMan NK2 micromanipulator.

S1.7 Embryo transfer

Two to six injected zygotes were transferred into the oviduct of each recipient sheep by using a TomCat catheter (Sovereign). Pregnancy was diagnosed at 50–60 days by ultrasound using a SonoVet 600 scanner (Medison) with a transrectal 5 MHz linear array probe. All these lambs were delivered naturally.

S1.8 Detection and Analysis of Indel Mutation in Lambs

Ear and muscle in hind leg were collected and digested in lysis buffer (10 μ M Tris-HCl, 0.4 M EDTA, 1% SDS, and 100 μ g·mL⁻¹ Proteinase K). The genomic DNA was extracted from lysate by phenol-chloroform and recovered by alcohol precipitation. SURVEYOR Nuclease Assay was performed as described above. PCR products with mutations detected by SURVEYOR Nuclease Assay were subcloned into pMD-19T vector. For each samples, at least 80 clones were picked up randomly and sequenced. Mutations were identified by comparing sequenced alleles with wild-type allele. Mutation types were analyzed and indel rates were calculated according to the sequencing results.

S1.9 Off-target Analyses

All potential off-targets sites with homology to the 23 bp sequence, 20 bp Spacer and 3 bp PAM (protospacer adjacent motif) were retrieved by a base-by-base scan of the whole sheep genome. Potential targets of sgRNAs were found using the principles of Mali et al. [1]. Considering the accuracy of the 12 nt 5' of PAM (NGG) sequence, four searched sequences (M)₁₂ A/T/C/G GG were generated. Exact matches to these searched sequences in the sheep genome were found using NCBI genome blast. Potential off-target sequences showing more than 5 nt mismatches were excluded. Six sites were selected for PCR amplifications using ear genomic DNA as templates, and SURVEYOR Nuclease Assays were performed to these products.

S2 Supplementary Figures

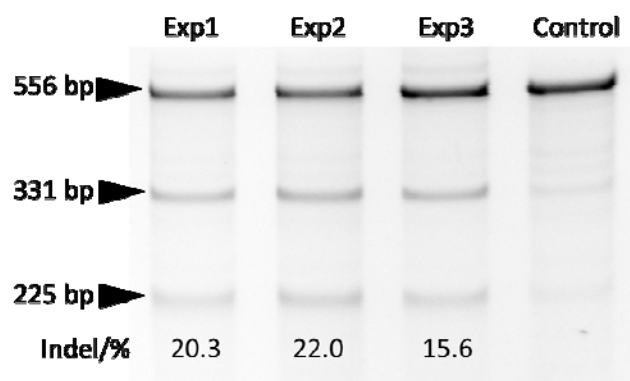


Fig. S1 Indel mutations were detected in sheep fibroblast cells. Three independent experiments were performed and the cleavage efficiency was estimated by the method described above

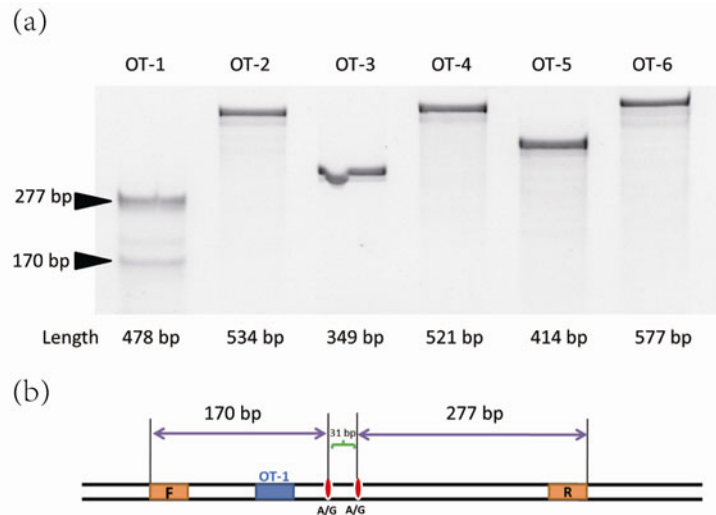


Fig. S2 (a) SURVEYOR Cleavage Assay results of six potential off-target sites; (b) The cleavage bands of off-target (OT) site1 were caused by two SNPs sites separated by 31 nt according to the sequencing results, which revealed one allele had AA genotype and the other GG corresponding to the two SNPs sites. Of the three expected cleavage bands, two longer bands (277 and 170 bp) were observed but the 31 nt one was too weak to detect

S3 Supplementary Tables

Table S1 The designed sgRNA target sites for *MSTN* gene

sgRNA	Target site (5' – 3') (PAM is shaded)	Oligonucleotide 1 (5' – 3')	Oligonucleotide 2 (5' – 3')
sgRNA- <i>MSTN</i>	ccctt gctgtactcc tacaagaatgtc	TAGG gacatcttt gtaggagtacagcaa	AAAC Ttgctgta ctcctacaagaatgtc

Note: The shaded nucleotides represent PAM sequence highlighted with yellow; the *Bbs* I-digested overhang sequences are highlighted in red and green.

Table S2 Primer list for amplification of templates for *in vitro* transcription

Components	Primer name	Primer sequence (5'–3')
Cas9	IVT-Cas9-F	agcagagctctctgctaactag
	IVT-Cas9-R	cacactggactagtggatccgag
sgRNA	IVT-sgRNA-F	ttaatacgactcactatagggacat
	IVT-sgRNA-R	aaaagcaccgactcgggtgcc

Table S3 Summary of generated *MSTN* mutant sheep via zygote injection of Cas9 mRNA/sgRNA

Batch of experiment	recipients	pronucleus embryos transferred	recipients conceived	Pregnancy/%	lambs	Mutant (% newborns)
1	28	130	16	57.1	22	16.9
2	27	83	15	55.6	13	15.7
Total	55	213	31	56.4	35	16.4

Table S4 Primer list for PCR amplification of the targeted sites

Primer name	Primer sequence (5'-3')
<i>MSTN</i> -sites-F	atgactccttgcggtaggagagt
<i>MSTN</i> -sites-R	gactgtagcatactctaggctta

Table S5 Off-target analyses

Name	Sequence (5'-3') (Seed + PAM)	Gene location	Strand	Coordinate	Primer sequence (5'-3')	Product length/bp
OT-1	gggtgggaggtacagcaaa AGG	38.5 kb of 5' MUSK	+	Chr2:12690547-12690566	F: CTCTTCACATCAGGTGGCTAATG R: GCATTTCTCGCCTGGAGCATCCTA	478
OT-2	tttcaggagtacagcaaa AGG	74.5 kb of 3' SEMA3D	+	Chr4:34718861-34718880	F: AGTGAACACCCAGGACTGATCTC R: GTTGACTTACAGTATCATGTTAG	534
OT-3	ttctgggagtacagcaaa AGG	58.9 kb of 3' LOC101113241	-	Chr7:61709924-61709943	F: AAGTGTGGCACATCACCACAGCC R: GACAAAGTCTGACTGTATATCAC	349
OT-4	gcttgggagtacagcaaa AGG	321 kb of 3' FLRT2	+	Chr7:93844155-93844174	F: TAGTCACCGACTCAATCAACATG R: GGTTCATATGAAAGACCTACAAGAC	521
OT-5	cttcaggagtacagcaaa AGG	196 kb of 3' BHLHE22	-	Chr9:41906337-41906356	F: ATGCAATAGACCATCATCTCGTG R: GCCAATTGACTGGCTGCTGCTCTG	414
OT-6	ttctgggagtacagcaaa TGG	Exon of JMJD1C	-	Chr35:19296454-19296473	F: TCTGCAAAAATGGAAAAGGTTGGAC R: GCCACTTCATTCAGCAGCTGTAG	577

S4 Supplementary sequences

S4.1 Seq. 1 pcDNA3.1(+)-Cas9

Full DNA sequence of Cas9 expression vector pcDNA3.1(+)-Cas9. The DNA sequence, containing *Cas9* sequence (underlined), a Kozak sequence (in red), 3 × Flag tag (in blue), and two different kinds of NLSs (highlight in green) was synthesized and subcloned into pcDNA3.1(+) vector through *Hind* III restriction site (in green). Start and stop codon were shown in bold.

GACGGATCGGGAGATCTCCCGATCCCCTATGGTGCACCTCTCAGTACAATCTGCTCTGA
TGCCGCATAGTTAAGCCAGTATCTGCTCCCTGCTTGTGTGTTGGAGGTCGCTGAGTAG
TGCGCGAGCAAAATTTAAGCTACAACAAGGCAAGGCTTGACCGACAATTGCATGAAG
AATCTGCTTAGGGTTAGGCGTTTTGCGCTGCTTCGCGATGTACGGGCCAGATATACGC
GTTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCA
TAGCCCATATATGGAGTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGA
CCGCCAACGACCCCGCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGC
CAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTT
GGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGT
AAATGGCCCGCCTGGCATTATGCCAGTACATGACCTTATGGGACTTTCCTACTTGGC
AGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACAT
CAATGGGCGTGGATAGCGGTTTACTCACGGGGATTCCAAGTCTCCACCCCAATTGAC
GTCAATGGGAGTTTGTGGTGGCACCAAAATCAACGGGACTTCCAATAATGTCGTAACA
ACTCCGCCCAATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAA
GCAGAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATAC
GACTCACTATAGGGAGACCCAAGCTGGCTAGCGTTTAAACTT**AAGCTT****GCCACCATG**
GACTATAAGGACCACGACGGCGACTACAAGGATCACGACATTGATTATAAAGACGAC
GACGATAAG**CCCAAGAAAAACGGAAGGTC**GATAAAAAATATAGCATCGGGCTCGA
CATTGGCACCAACTCCGTGGGCTGGGCTGTGATCACAGACGAGTACAAAGTCCCCAG
CAAGAAGTTCAAGGTCCTCGGGAACACCGATAGGCACTCCATCAAGAAGAATCTGAT
TGGCGCCCTGCTCTTTGACAGCGGCGAGACAGCCGAAGCTACCCGGCTGAAAAGGAC
AGCTCGGCGGAGGTATACAAGGCGGAAGAATCGGATTTGCTACCTGCAGGAAATCTT
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GAAGTGGCCTATCATGAGAAGTACCCACCATCTACCACCTCCGGAAGAACTCGTC
GACAGCACCGACAAGGCCGATCTGAGGCTGATTTACCTCGCTCTGGCCACATGATTA
AGTTTCGGGGGCATTTTCTCATTGAAGGCGACCTGAACCCCGACAATTCCGATGTGCA
CAAGCTGTTTCATCCAGCTGGTCCAGACCTATAACCAGCTCTTCGAGGAGAACCCCATC
AACGCTCCGGGGTCGACGCCAAAGCTATCCTGTCCGCCAGGCTGAGCAAGTCCAGG
CGGCTCGAAAACCTCATTGCCAGCTCCCCGGCGAGAAGAAGAATGGCCTGTTTGGG
AACCTGATTGCTCTCAGCCTCGGGCTCACCCCTAACTTCAAGTCCAACCTCGACCTGG
CTGAGGACGCCAAACTCCAGCTGTCCAAGGATACCTACGACGATGACCTCGATAACC
TGCTGGCCAGATCGGCGACCAGTACGCCGATCTGTTTCTCGCTGCCAAAAACCTGAG
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AAAAAAA **TGATGAAAGCTTGGTACCGAGCTCGGATCCACTAGTCCAGTGTGGTGGG**
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CTTTTCTGGATTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATA
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AGAAGTAAGTTGGCCGACAGTGTATCACTCATGGTTATGGCAGCACTGCATAATTCTC
TACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTC
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AATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGG
GGCGAAAACCTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCG
TGCACCCAACCTGATCTTCAGCATCTTTTACTTTTACCAGCGTTTCTGGGTGAGCAAAA
ACAGGAAGGCAAAATGCCGCAAAAAGGGAATAAGGGCGACACGGAAATGTTGAAT

ACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTATCAGGGTTATTGTCTCATGA
GCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCGCGCACAT
TCCCCGAAAAGTGCCACCTGACGTC

S4.2 Seq. 2 pMD-19T-sgRNA

Full DNA sequence of sgRNA expression vector pMD-19T-sgRNA. T7 promoter is in bold. The pair of *Bbs* I restriction sites are indicated in green. The sequence highlighted in yellow is replaced with the annealed oligonucleotides in the following experiment to create customized sgRNA and the remainder of sgRNA is underlined.

TCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGG
TCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAG
CGGGTGTGGCGGGTGTGCGGGCTGGCTTAACTATGCGGCATCAGAGCAGATTGTAC
TGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACC
GCATCAGGCGCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGC
GGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAA
GTTGGGTAACGCCAGGGTTTTCCAGTCACGACGTTGTA AACGACGGCCAGTGAAT
TCGAGCTCGGTACCCGGGGATCCTCTAGAGATt**TAATACGACTCACTA****TAGGACGTC**
TTCAACACAAGAAGACACGTTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGT
CCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTtATCGTCGACCTGCAG
GCATGCAAGCTTGGCGTAATCATGGTCATAGCTGTTTCTGTGTGAAATTGTTATCCG
CTCACAATCCACACAACATACGAGCCGGAAGCATAAAGTGTAAGCCTGGGGTGCC
TAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCCGTTTCCAGTCGG
GAAACCTGTGTCGAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTT
TGCATTGCGGCTCTTCCGCTTCTCGCTCACTGACTCGCTGCGCTCGGTCTGTTCCG
CTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCA
GGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCG
TAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCAC
AAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCA
GGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCG
GATACCTGTCCGCTTTCTCCCTTCGGGAAGCGTGCGCTTTCTCATAGCTCACGCTGT
AGGTATCTCAGTTCGGTGTAGGTCGTTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCC
CCGTTACGCCCAGCCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGT
AAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAG
GTATGTAGGCGGTGCTACAGAGTTCCTGAAGTGGTGGCCTAACTACGGCTACACTAG
AAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTT
GGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTTGTTTGCA
AGCAGCAGATTACGCGCAGAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTA
CGGGGTCTGACGCTCAGTGGAACGAAACTCACGTTAAGGGATTTTGGTCATGAGAT
TATCAAAAAGGATCTTACCTAGATCCTTTTAAATTA AAAATGAAGTTTTAAATCAAT
CTAAAGTATATATGAGTAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCA
CCTATCTCAGCGATCTGTCTATTTTCGTTCCATAGTTGCCTGACTCCCCGTCGTGTA
GATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCG
AGACCCACGCTACCCGGCTCCAGATTTATCAGCAATAAACAGCCAGCCGGAAGGGC

CGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGC
CGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTG
CTACAGGCATCGTGGTGTACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCC
CAACGATCAAGGCGAGTTACATGATCCCCATGTTGTGCAAAAAAGCGGTTAGCTCC
TTCGGTCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTA
TGGCAGCACTGCATAATTCTTACTGTGTCATGCCATCCGTAAGATGCTTTTCTGTGACT
GGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTT
GCCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCA
TCATTGGAAAACGTTCTTCGGGGCGAAAACCTCTCAAGGATCTTACCGCTGTTGAGATC
CAGTTCGATGTAACCCACTCGTGCACCCAACCTGATCTTCAGCATCTTTTACTTTCACCA
GCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAAATGCCGCAAAAAAGGGAATAAGG
GCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTA
TCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACA
ATAGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATT
ATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTC

Reference

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